

Determination of Nitrite Plus Nitrate and Malondialdehyde in Human Plasma: Analytical Performance and the Effect of Smoking and Exercise

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The aim of this study was to evaluate the analytical performance and clinical usefulness of spectrophotometric assays for the measurement of the plasma levels of nitrite plus nitrate (NOx), and malondialdehyde (MDA), as an index of nitric oxide release and lipid peroxidation, respectively. We studied 30 healthy sedentary volunteers, 12 endurance athletes and 12 regular heavy smokers. The lower limit of quantification for plasma NOx concentration was 1 $\mu\text{mol/l}$, and linearity was observed from 1 to 40 $\mu\text{mol/l}$ of NOx concentration. Variation in replicate samples within or between days was always below 5%. NOx levels were significantly higher in athletes compared to both control subjects and smokers ($p < 0.05$ and $p < 0.001$, respectively), as well as in healthy subjects compared to smokers ($p < 0.05$). The analytical limit of quantification for plasma MDA concentration was 0.03 $\mu\text{mol/l}$, and linearity was observed from 0.03 to 20 $\mu\text{mol/l}$ of MDA concentration. Variation in replicate samples within or between runs was $< 5\%$. Mean MDA concentration was significantly higher in smokers compared to control subjects and athletes ($p < 0.001$). A significant inverse relationship ($p < 0.001$) was observed when comparing NOx with MDA ($r = -0.49$) or LDL levels ($r = -0.30$) in the total population. The assays evaluated in this study proved to be sensitive, specific and practicable, and therefore suitable for routine application in clinical chemistry laboratories and/or physiopathological studies involving human blood samples. Clin Chem Lab Med 2002; 40(8):802–809

Key words: Nitric oxide; Malondialdehyde; Endothelial function.

Abbreviations: BHT, butylated hydroxytoluene; GSH, glutathione; GSSG, oxidized glutathione; HDL, high density lipoprotein; LDL, low density lipoprotein; MDA, malondialdehyde; NO, nitric oxide; NOx, nitrite and nitrate; $\text{VO}_{2\text{max}}$, maximal oxygen consumption.

Introduction

Nitric oxide (NO) release and oxidative reactions are of central importance in the maintenance of vascular

homeostasis and the progression of vascular disease. In particular, the balance between them may have important clinical implications in the processes such as atherosclerosis, where oxidant stress appears to play a pivotal role in the onset and progression of vascular lesions (1).

In these pathological settings, the NO radical molecule can inhibit lipid peroxidation by scavenging propagatory lipid peroxy radicals, but it also behaves as a potent pro-oxidant producing peroxynitrite in the presence of superoxide anion (2). For these reasons, attention has been increasingly focused on the possibility of monitoring NO synthesis and release and the evaluation of free radical generation in tissues and biological fluids. However, direct quantitative measurement of these processes is extremely difficult and not applicable *in vivo* (3, 4).

Plasma malondialdehyde (MDA) levels, evaluated as a marker of lipid peroxidation and oxidative stress, and plasma levels of inorganic nitrites and nitrates (NOx), representing the stable and final metabolites of the NO metabolic pathway, can be measured with colorimetric assays (3, 5). These methods do not require specific or complex instrumentation and are not expensive or time-consuming. Consequently, the colorimetric assay for MDA and NOx may be considered the best methods to use in clinical studies aiming to evaluate the involvement of NO and oxidative stress in various pathophysiological conditions and to assess the effect of pharmacological treatments on these variables. However, no general agreement exists on their use, and the views on the specificity of these tests differ (3, 6).

The present study was designed to evaluate the analytical characteristics of these colorimetric assays. The other aim of our study was to verify the clinical reliability of these methods. In particular, we evaluated whether the MDA and NOx assays demonstrate different patterns of oxidative stress and NO bioavailability *in vivo* in regular smokers and endurance athletes, compared to sedentary healthy subjects.

Subjects and Methods

Subjects

We studied 30 healthy sedentary volunteers (21 males, 9 females, mean age 29.2 ± 4.1 years), 12 male endurance athletes (mean age 32.8 ± 5 years) and 12 smokers (6 males, 6 females, mean age 32.7 ± 4.8 years).

All subjects were non-obese and had arterial blood pressure in the normal range (Table 1); they were free from acute diseases, as determined by an interview with a clinician. Furthermore, they denied the use of any medication during the 4 weeks prior to the study. All of them had normal values for the

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Table 1 Clinical characteristics of the studied subjects.

	Sedentary volunteers (n=30)	Athletes (n=12)	Smokers (n=12)
Age (years)	29.20±4.1	32.8±5	32.70±4.8
Body-mass index (kg/m ²)	23.12±2.3	23.8±3.3	24.80±3.6
Systolic blood pressure (mmHg)	122.50±11.2	123.5±6.8	125.10±6.6
Diastolic blood pressure (mmHg)	78.80±6.3	76.7±4	79.40±4.3
Heart rate (beats/minute)	69.10±7.3	60.0±6.6	68.00±5.6
Total cholesterol (mmol/l)	4.60±0.5	4.9±0.6	5.00±0.7
HDL-cholesterol (mmol/l)	1.30±0.3	1.7±0.4*	1.40±0.4
LDL-cholesterol (mmol/l)	2.90±0.4	3.0±0.5	3.24±0.6
VO _{2max} (ml/kg min)	42.20±2.8	66.6±3.6**	36.60±3.9

*p<0.05 vs. sedentary group; **p<0.01 vs. sedentary group and smokers

main plasma parameters (including creatinine, urea nitrogen, glucose, uric acid, albumin, enzymes, electrolytes, and hemoglobin), normal erythrocyte and leukocyte count and normal urine analysis.

Athletes (triathletes, long distance runners and cyclists) were selected on the basis of maximal oxygen consumption (VO_{2max}) above 60 ml/min/kg, assessed during a graded exercise test performed on a cycle ergometer. The sedentary subjects performed no physical activity and had a VO_{2max} below 45 ml/min/kg (7). Athletes were requested to avoid physical activity for 24 h before the day of the study.

Smokers had a smoking history of 1–2 cigarette packs/day (daily cigarette consumption: mean 22.5±8.4) for at least 5 years.

All participants fasted for at least 12 h before the study period, were requested to adhere to a low-nitrate diet (namely, to exclude foods containing high concentration of nitrate such as cured meat, fruits and, in particular, leafy green vegetables) for 72 h before collection of blood samples. Women were studied on the 4th to the 7th day of their menstrual cycle.

Informed consent was obtained from each subject entering the study, and the experimental protocol was approved by the local Hospital Ethics Committee.

Blood sampling

All subjects were examined at 9.00 am in a quiet, air-conditioned room with temperature maintained at 22–24°C. After introduction of an indwelling cannula (Artsana SPA, Grandate, Como, Italy) into the left antecubital vein, each subject was allowed to rest in a supine position for at least 15 min before blood collection. Venous blood samples were collected into tubes containing dipotassium ethylenediaminetetraacetate (EDTA). Samples for MDA determination were kept on ice and centrifuged within 15 min after blood collection at 2500 g for 15 min. Butylated hydroxytoluene (BHT) (5 mmol/l) was then added to plasma samples to prevent further oxidation.

Analytical methods

Plasma concentrations of total cholesterol, high density lipoprotein (HDL)-cholesterol and triglycerides were determined by standard laboratory methods. The concentration of low density lipoprotein (LDL)-cholesterol was calculated using the Friedewald equation, and expressed as mmol/l.

NOx assay

Plasma samples were stored at –80°C for less than 2 weeks before analysis. At the time of NOx assay, plasma samples were ultrafiltered (30 kDa molecular weight cut-off) and cen-

trifuged at 1000 g for 60 min in order to remove hemoglobin, which is known to interfere with spectrophotometric measurements. NOx concentration in different dilutions of plasma ultrafiltrate was determined by using a reagent kit (Cayman, Ann Arbor, USA) based on the Griess reaction (8), which consists of three main steps: 1) enzymatic conversion of nitrate to nitrite by means of nitrate reductase in the presence of 5 mmol/l NADPH; 2) incubation with Griess reagent (0.1% N-(1-naphthyl)ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% H₃PO₄) for 10 min at 25°C to convert nitrite into a chromophore compound; 3) quantitative estimation of nitrite concentration by spectrophotometric measurement of the absorbance at 540 nm (ETI-system, Sorin Biomedica, Verelli, Italy). Standards for calibration curves were prepared with nitrate and taken through the full assay procedure. The results were expressed as µmol/l of NOx.

MDA assay

Plasma samples were stored at –80°C for less than 1 week before analysis. MDA was measured by a colorimetric assay based on the reaction among one molecule of MDA and two molecules of N-methyl-2-phenylindole at 45°C to yield a stable chromophore compound. Subsequently, turbid samples were centrifuged at 15000 g for 10 min to obtain a clear supernatant with maximal absorbance at 586 nm (Oxis International, Inc., Portland, USA). Since MDA itself is not stable, the standards used for the construction of the calibration curves were prepared as diethylacetals and taken through the full assay procedure. The acetals were hydrolyzed during the acid incubation at 45°C generating MDA. The results were expressed as µmol/l of MDA.

Statistical analysis

Data are expressed as mean±SD unless otherwise stated. The significance of differences between plasma NOx concentration and plasma MDA levels was evaluated by one-way ANOVA, and the Bonferroni correction was applied in case of multiple comparisons among the three groups of subjects. Differences were considered statistically significant at p<0.05. Linear regression analysis was used to assess the relationships between plasma concentrations of LDL-cholesterol, MDA and NOx.

Results

Demographic and clinical characteristics of the three studied groups are shown in Table 1. Data from ath-

letes, sedentary subjects and smokers were comparable in terms of age and major clinical parameters. Trained individuals, in accordance with the inclusion criteria, showed an increased $\text{VO}_{2\text{max}}$ compared to sedentary subjects (Table 1).

NOx assay

Analytical performance

The limit of quantification, defined as the concentration corresponding to the mean value of 14 determinations of the zero calibrator +2 SD, was 1 $\mu\text{mol/l}$. The assay was found to be linear from 1 to 40 $\mu\text{mol/l}$ of nitrate concentration ($r=0.99$, $p<0.001$) (Figure 1, panel A). The dilution of plasma samples (to 1:10) did not significantly modify the NOx results. Both within-run and between-run assay imprecision, tested by repeated measurements of 11 plasma samples with NOx concentration between 29.8 and 59.1 $\mu\text{mol/l}$, were always below 5%.

Results of the analytical recovery tests are shown in Tables 2 and 3. In order to evaluate the recovery of both analytical steps (*i.e.* ultrafiltration and colorimetric analysis), known amounts of NOx were added (to plasma samples to be assayed; before ultrafiltration (Table 2), as well

as to ultrafiltered plasma (Table 3). A good analytical recovery was observed for both the colorimetric analysis alone ($104.9\pm2.5\%$) and the total assay ($99.7\pm7.1\%$).

Clinical results

Plasma samples, collected from five healthy volunteers at different times throughout the day (9 am, 1 pm,

Table 2 The measurement of nitrate and nitrite. Analytical recovery excluding the ultrafiltration step.

NOx concentration ($\mu\text{mol/l}$)				Recovery (%)
Initial	Added	Expected	Measured	
5.5	10	15.5	15.8	102.3
5.5	15	20.5	21.1	103.0
5.5	20	25.5	27.5	107.9
5.5	25	30.5	31.2	102.3
5.5	30	35.5	38.0	107.0
5.5	35	40.5	42.2	104.3
5.5	40	45.5	48.9	107.5

Known NOx concentrations were added to an ultrafiltered sample with fixed known concentration.

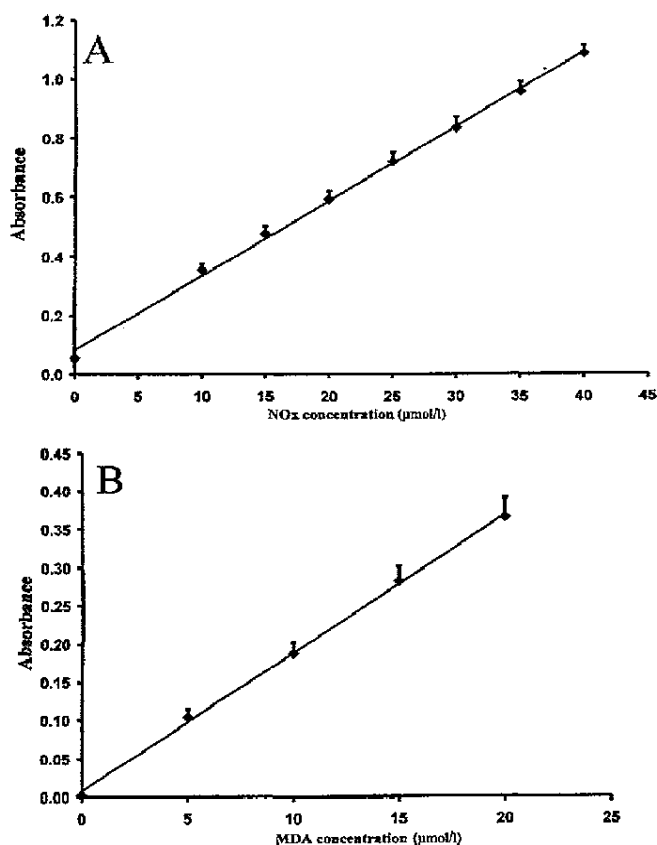


Figure 1 Standard curve (A) for the measurement of nitrate plus nitrite ($n=14$; $r=0.999$, $p<0.001$) at 540 nm and (B) for the measurement of malondialdehyde ($n=10$; $r=0.999$, $p<0.001$) at 586 nm.

Table 3 The measurement of nitrate and nitrite. Analytical recovery including the ultrafiltration step.

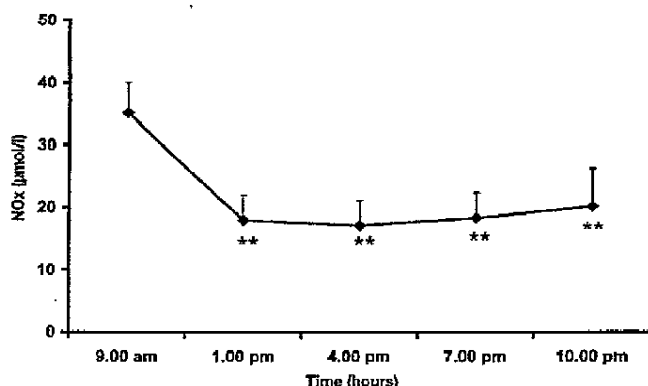
NOx concentration ($\mu\text{mol/l}$)				Recovery (%)
Initial	Added	Expected	Measured	
11	10	21	18.8	89.9
9.6	8.75	18.35	17.6	95.9
8.4	7.5	15.9	15.2	95.5
6.8	6.2	13	13.4	102.4
5.5	5	10.5	11.8	105.7
2.7	2.5	5.2	5.7	108.8

Known NOx concentrations were added to plasma before ultrafiltration and then diluted and assayed.

Table 4 The measurement of malondialdehyde. Analytical recovery.

MDA concentration ($\mu\text{mol/l}$)				Recovery (%)
Initial	Added	Expected	Measured	
0.135	0	0.135	0.13	96.5
0.135	0.5	0.635	0.54	85
0.135	1	1.135	0.99	87.2
0.135	1.5	1.635	1.54	94.1
0.135	2	2.135	1.97	92.2

Known MDA concentrations were added to a sample with fixed known concentration.

**Figure 2** Plasma levels of NOx in five subjects at different times of the day. ** $p < 0.001$ vs. 10 am.

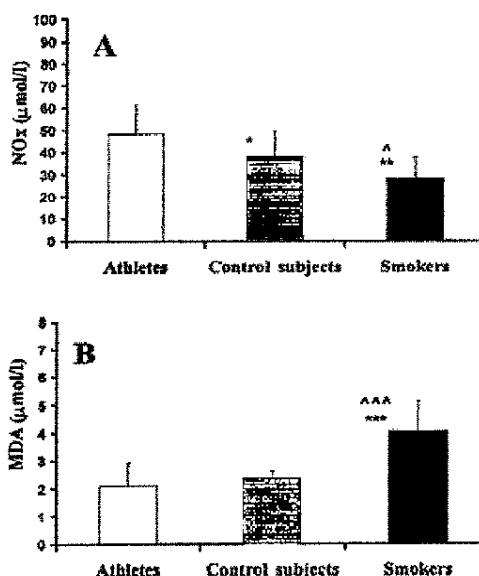
4 pm, 7 pm, 10 pm), were assayed; the results are reported in Figure 2. These data indicate that NOx levels significantly declined from 9 am to 1 pm, without further changes during the rest of the day.

Mean plasma NOx levels were significantly higher in athletes ($48.5 \pm 13.2 \mu\text{mol/l}$) compared to both control subjects ($38.2 \pm 11.5 \mu\text{mol/l}$, $p < 0.05$) and to smokers ($28.2 \pm 9.4 \mu\text{mol/l}$, $p < 0.001$). Furthermore, the mean value observed in healthy subjects was significantly ($p < 0.05$) different from that observed in smokers (Figure 3, panel A).

MDA assay

Analytical performance

The limit of quantification, defined as the concentration corresponding to the mean value of 10 determinations of the zero calibrator +2 SD, was $0.03 \mu\text{mol/l}$. The assay was found to be linear from 0.03 to $20 \mu\text{mol/l}$ ($r = 0.99$, $p < 0.001$; Figure 1, panel B). Both within-run and between-run assay imprecision, tested by repeatedly assaying six plasma samples with different MDA concentrations (from 1.81 to $3.19 \mu\text{mol/l}$), were always below 5%. The dilution of plasma samples (to 1:20) did not significantly modify the estimation of plasma MDA levels. In order to evaluate the recovery, known amounts of MDA were added to five different aliquots of the

**Figure 3** Plasma levels of (A) NOx and (B) MDA in athletes, sedentary healthy subjects and smokers. The values are expressed as mean \pm SD. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$ vs. athletes; ^ $p < 0.05$, ^^ $p < 0.001$ vs. control subjects.

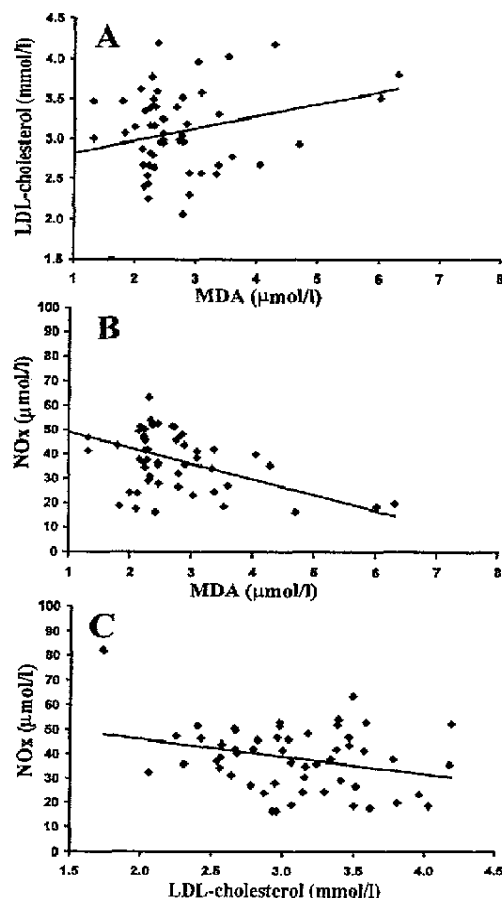


Figure 4 The relationship between (A) plasma MDA and LDL-cholesterol ($r=0.3$, $p<0.05$), (B) plasma levels of MDA and NOx ($r=-0.5$, $p<0.001$) and (C) plasma LDL-cholesterol and NOx ($r=-0.3$, $p<0.001$) in the entire study population.

same plasma sample. The results obtained are reported in Table 4; an analytical recovery of $91.0 \pm 4.8\%$ was observed.

Clinical results

Mean plasma MDA concentration was significantly higher in smokers (4.1 ± 1.1 $\mu\text{mol/l}$) compared to control subjects (2.4 ± 0.26 $\mu\text{mol/l}$, $p<0.001$), as well as to athletes (2.1 ± 0.8 $\mu\text{mol/l}$, $p<0.001$) (Figure 3, panel B).

Relationship between NOx, MDA and LDL-cholesterol in the entire studied population

A direct linear relationship was found between the circulating levels of MDA and LDL-cholesterol in the entire studied population ($r=0.3$, $p<0.05$; Figure 4, panel A). Furthermore, a significant inverse linear relationship was observed when comparing plasma NOx concentrations with plasma concentrations of MDA

($r=-0.49$, $p<0.001$) or LDL-cholesterol ($r=-0.3$, $p<0.001$) in the entire study population (Figure 4, panels B and C).

Discussion

Since the half-life of NO *in vivo* is very short, a direct quantitative estimation of its basal production is very difficult. In addition, different methodological approaches available retain different characteristics due to measurement of NO itself or its final metabolites, instantaneously or cumulatively. Chemiluminescence represents a sensitive and accurate method, although it requires careful sample preparation to reduce the loss of NO (9). Electron paramagnetic resonance is not particularly sensitive and is not widely used to quantify NO (9). In addition, these techniques require expensive, specialized equipment and considerable expertise to perform the assay; consequently, they are not appropriate for the clinical routine. NO-sensitive electrodes, recently manufactured, can be inserted directly into the sample and permit to monitor NO synthesis in real time, and progressing reactions over time (10). Because of their differential nature, electrochemical measurements do not permit the evaluation of basal NO production. In addition, these sensors, often modified to suit individual applications, are still being validated.

Spectrophotometric assays available for the indirect assessment of NO production are based on very different principles: the Griess reaction, which quantifies NOx after its reduction, and the hemoglobin method, which provides an estimation of oxyhemoglobin to methemoglobin due to NO (9). Hemoglobin method provides a highly sensitive method that does not require specialized apparatus to measure NO release in cell or tissue preparations or in perfusion experiments, and is particularly suitable for kinetic studies. However, its specificity is relative, because peroxynitrite, in addition to NO, can cause absorbance changes (11). In addition, care must be taken with regard to time, pH and temperature, because even small variations in these parameters can greatly influence the final result.

Since inorganic nitrites and nitrates are stable end-metabolites of NO, the assessment of plasma NOx concentration has become the main procedure in which clinical studies can attempt to assess changes in NO production *in vivo* (12–14). This method is the only one available in which the presence of oxygen or superoxide anion in the sample, as well as NO instability, is not a problem.

Our data indicate that the spectrophotometric methods for NOx assay are simple, cheap and reproducible. Furthermore, our results on normal subjects and athletes are similar to those previously reported (15). It is important to emphasize that several analytical, physiological, and clinical factors should be taken into account in order to set up an assay method for NOx and evaluate its results. Indeed, some differences may be found when comparing NOx plasma levels from different studies, even when only samples from healthy subjects

(reference values) are considered (15–17). Confounding factors which may explain these discrepancies are: differences in gender, diet, physical activity, smoking, as well as the time of blood sampling (18–21). In addition, the contribution of the endothelial NO synthase gene may be partly responsible for the differences (22, 23).

Since all subjects enrolled in the present study fasted and followed a low-nitrate diet, potential differences in dietary composition or nitrate intake should not account for NO_x variation. Since the time of blood sampling can affect plasma NO_x concentration, as suggested by the significant variation in plasma NO_x concentration throughout the day (Figure 2), we decided to collect blood only from subjects at rest and at the same time of day (9.00 am). Finally, our data were obtained from females during the early follicular phase of the menstrual cycle, when blood levels of estrogens are still relatively low and their possible effects on endothelial function are minimized.

The direct measurement of free radical generation using electron spin resonance represents an accurate method applicable to experimental models, but it is not suitable for use in humans (24). *Ex vivo* spin trapping has been utilized in clinical settings but its use is limited by the *ex vivo* generation of secondary oxidative species (25). Isoprostanes, derived from arachidonic acid, have been recently indicated as markers of antioxidant deficiency and oxidative stress (26) but are still questioned for their specificity and accuracy. In addition, other mechanisms leading to molecular changes in plasma proteins may help to evaluate the excess free radical generation. Reactive nitrogen species can react with tyrosine residues of proteins; thus, protein nitration may represent a marker for nitrating molecules and it can be used as a marker of oxidative protein damage (27).

However, the methods employed to evaluate these molecules are often elaborate and this limits their application in routine clinical diagnosis. Indeed, the measurements of 8-isoprostane in plasma by immunoenzymatic method require sample purification that takes hours (28). Nitrotyrosine levels are determined in previously filtered plasma samples by HPLC (29), or by Western blot and ELISA methods (30).

MDA, generated by the decomposition of polyunsaturated fatty acid peroxides, is largely used as an index of oxidative stress in cells and tissues (6), and MDA levels correlate closely with those of isoprostane (31).

At present, several commercial kits are available that offer a straightforward and rapid procedure for evaluating MDA in plasma, requiring equipment commonly found in biochemical laboratories. However, it is still a matter of debate whether the assay of this molecule represents a reliable method for the determination of free radical generation suitable for clinical studies (32). Analytical aspects and the assay performance could explain some of the discrepancies reported using different methods to estimate free radical generation (32). In particular, the measurement of MDA can be greatly affected by blood collection and storage procedures.

Indeed, unless assayed immediately, samples must be kept on ice after collection and centrifuged at 4°C. Furthermore, BHT must also be added to samples to a final concentration of 5 mmol/l; if no antioxidant is added, new lipid peroxidation can occur and bias will result. Unless assayed immediately, samples should be frozen at –80°C to prevent both loss of MDA and new sample oxidation. After the incubation at 45°C, turbid samples may not clear upon centrifugation. In this case, a dilution of the sample to obtain clear samples is possible without any loss of accuracy (one can dilute 200–50 µl in 1000 µl of total reaction mixture). Finally, the used sample should not be re-frozen.

Exercise, smoking habit, nitric oxide and oxidative stress

Our findings show that aerobic physical training and plasma NO availability are positively associated and thus are in accordance with other recent results (20, 33). Enhanced blood flow due to exercise increases laminar shear stress. It is well-known that shear stress upregulates the expression and activity of endothelial nitric oxide synthase (eNOS) in the endothelial cells (34, 35).

Another possible mechanism by which exercise could improve NO availability might be related to the prevention of oxidative stress. In fact, if at high NO concentrations, resulting from the activation of inducible NOS, and in the presence of oxidant molecules with which it can react, NO promotes nitration or oxidative reactions; at physiological levels NO protects endothelium against oxidant species including oxLDL (36). Accordingly, recent data indicate that physical activity can improve endothelial-dependent vasodilation and prevent the age-related NO breakdown in elderly subjects, by a mechanism essentially related to its antioxidant activity (37).

In addition, the effects of NO on antioxidant molecules, such as glutathione (GSH), and on enzymes related to its metabolism, are now recognized to be important. GSH protects the endothelium by acting as a physiological free radical scavenger. Recent data indicate that NO, at a physiological level of endothelial production, induces the synthesis of GSH through a mechanism involving γ-glutamylcysteine synthase and γ-glutamyl transpeptidase (36). In addition, chronic N(omega)-nitro-L-arginine methyl ester L-NAME (NOS inhibitor) treatment in rats has proved to increase MDA levels and cause depletion of NO, NOS activity, NOS protein expression and to decrease GSH/GSSG (GSSG, oxidized glutathione) ratio (38). Chronic training reduced MDA and improved both NO and antioxidant molecules, while the combination of physical exercise and L-NAME induces normalization of antioxidant enzyme activity as well as protein expression (38). Thus, the existence of this cooperative interaction between NO and GSH may be crucial for the protection of endothelium against oxidative stress. Further studies are required in this field.

Cigarette smoke (especially the gas phase) contains

high amounts of free radicals and pro-oxidants (39). MDA levels, measured in erythrocytes and evaluated as indicators of oxidant status, were significantly higher in smokers compared to non-smokers (40). Thus, smokers appear to be particularly susceptible to the activity of oxygen-derived free radicals, and plasma levels of 8-isoprostane in smokers are increased (41). A blunted response to endothelium-dependent vasodilators has been revealed in chronic smokers, which improves with antioxidant treatment (42). In addition, the vasoconstrictor response to the inhibition of NO by N^G-monomethyl-L-arginine (L-NMMA) is reduced in long-term smokers, suggesting impaired basal NO-mediated vasodilation (43).

In line with these results, our data show a marked reduction in the plasma levels of NOx in smokers compared to control subjects. Furthermore, low levels of plasma NOx are associated with significantly higher levels of plasma MDA in heavy smokers. Finally, a significant inverse relationship between NOx and MDA or plasma LDL-cholesterol plasma levels was found in the combined groups of studied subjects. These data suggest that although the mechanism of smoking-related endothelial dysfunction is certainly multifactorial, this effect may be partly explained by the sequence of events including impaired NO production or enhanced NO degradation resulting from oxygen-derived free radical production which would then lead to enhanced lipid peroxidation and MDA production (38, 40).

Thus, if decreased NO bioavailability in smokers supports the existence of a link between NO activity and oxidative stress, regular physical activity appears to represent a physiologic antioxidant.

Conclusions

The evaluation of nitrite plus nitrate and MDA in plasma samples represents sensitive, specific and practicable indicators of NO availability and oxidative stress, respectively. Both methods are quick and simple and can be used to process a large number of samples. For this reason, these methods may be considered suitable for routine application in clinical chemistry laboratories and for use in physiopathological studies involving human blood samples. However, particular care must be taken with collection and storage of plasma samples.

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